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Analysis of Ebola virus and VLP release using an immunocapture assay

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Abstract

Ebola virus (EBOV), an emerging pathogen, is the causative agent of a rapidly progressive hemorrhagic fever with high mortality rates. There are currently no approved vaccines or treatments available for Ebola hemorrhagic fever. Standard plaque assays are currently the only reliable techniques for enumerating the virus. Effective drug-discovery screening as well as target identification and validation require simple and more rapid detection methods. This report describes the development of a rapid ELISA that measures virus release with high sensitivity. This assay detects both Ebola virus and EBOV-like particles (VLPs) directly from cell-culture supernatants with the VP40 matrix protein serving as antigen. Using this assay, the contribution of the EBOV nucleocapsid (NC) proteins in VLP release was determined. These findings indicate that a combination of NC proteins together with the envelope components is optimal for VLP formation and release, a finding that is important for vaccination with Ebola VLPs. Furthermore, this assay can be used in surrogate models in non-biocontainment environment, facilitating both basic research on the mechanism of EBOV assembly and budding as well as drug-discovery research.

Keywords: Ebola; VLP; VP40; NP; Filovirus; Diagnostic; ELISA

1. Introduction

Ebola virus (EBOV), a member of the family Filoviridae, is a highly pathogenic virus capable of aerosol infection. It is classified by the Centers for Disease Control and Prevention (Atlanta, GA) as a select agent requiring biosafety level 4 containment for handling (Feldmann et al., 2003). Ebola hemorrhagic fever is of serious concern as mortality rates have approached 90% and periodic outbreaks of the disease in sub-Saharan Africa show an upward trend in recent years (http://www.who.int/disease-outbreak-news/disease_indices/ebol_index.html). Additionally, the potential for filoviruses as a biological weapon is of grave concern to the human population. Our current understanding of basic mechanisms of the filoviral lifecycle and how filoviruses interact with cellular machinery is lagging behind when compared to many other virus families. This is largely due to the restrictions inherent to biosafety level 4 (BSL4) containment. Central to these studies is a surrogate model for viral replication, assembly, and budding, as well as a rapid assay for measurement of viral or non-infectious particle release.

Two surrogate models were developed recently for the study of filoviruses. One model is based on the ability of VP40 and GP of EBOV (Bavari et al., 2002; Jasenosky et al., 2001; Panchal et al., 2003; Timmins et al., 2001) and Marburg (Swenson et al., 2004) to spontaneously form filamentous virus-like particles (VLPs). Striking morphological similarity of VLPs to the authentic virus (Bavari et al., 2002; Swenson et al., 2004; Timmins et al., 2001; Warfield et al., 2004a) suggests that the process of VLP formation probably closely resembles that of the live virus, making this system an ideal model for studying filoviral assembly and release.

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14. ABSTRACT
Ebola virus (EBOV), an emerging pathogen, is the causative agent of a rapidly progressive hemorrhagic fever with high mortality rates. There are currently no approved vaccines or treatments available for Ebola hemorrhagic fever. Standard plaque assays are currently the only reliable techniques for enumerating the virus. Effective drug-discovery screening as well as target identification and validation require simple and more rapid detection methods. This report describes the development of a rapid ELISA that measures virus release with high sensitivity. This assay detects both Ebola virus and EBOV-like particles (VLPs) directly from cell-culture supernatants with the VP40 matrix protein serving as antigen. Using this assay, the contribution of the EBOV nucleocapsid (NC) proteins in VLP release was determined. These findings indicate that a combination of NC proteins together with the envelope components is optimal for VLP formation and release, a finding that is important for vaccination with Ebola VLPs. Furthermore, this assay can be used in surrogate models in non-biocontainment environment, facilitating both basic research on the mechanism of EBOV assembly and budding as well as drug-discovery research.

15. SUBJECT TERMS
filovirus, Ebola, VLP, VP40, NP, ELISA, method, diagnosis

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Recently, this system was combined successfully with reverse genetics making it possible to study the assembly process in the presence of packageable RNA (Watanabe et al., 2004). A second, widely used model is based on pseudotyping of other viruses with EBOV glycoprotein. However, the applicability of this model is limited to the study of viral entry, and even in this area, the results have to be interpreted cautiously due to a different morphology of the pseudotyped virus from the wild type virus. Therefore, the VLP model is currently the only non-infectious model for studying the filoviral assembly and budding. VLPs are also promising vaccine candidates as demonstrated recently where EBOV and Marburg VLPs could induce a fully protective immune response in rodents (Warfield et al., 2003, 2004b).

VP40 is the filoviral matrix protein and is the most abundant protein incorporated into the mature virion (Feldmann and Klenk, 1996). The crystal structure of EBOV VP40 shows that the protein consists of two domains both folding into similar β-sandwich structures connected by a flexible linker region that allows the two domains to rotate into different conformations (Dessen et al., 2000). Conformational changes in monomeric VP40 are thought to play an important role in mediating coordinated oligomerization and membrane localization of the protein, a process that is critical in the formation of filamentous VLP structure (Panchal et al., 2003; Scianimanico et al., 2000). VP40 also contains three potential viral late domains that seem to play a role in viral egress for some retroviruses, rhabdoviruses, and filoviruses (Freed, 2002; Licata et al., 2003), although these motifs are not entirely conserved in Marburg virus strains. While VP40 is the driving force for filamentous particle formation, recent reports indicate that other viral proteins such as glycoprotein (GP), and nucleoprotein (NP) enhance the VLP release and are incorporated into VLPs (Bavari et al., 2002; Licata et al., 2004). These findings underscore the application of the VLP as a model for investigating filovirus assembly and budding, and the role ofcellular factors in this process. Furthermore, this experimental model provides an excellent system for anti-filovirus drug-discovery.

Effective application of the VLP model requires a rapid particle release assay. Such an assay could also replace or be complementary to the conventional viral plaque assays for virus detection, which require more than a week to accomplish. Previous VLP release assays relied on Western blot analysis, a technique that is poorly quantitative and not amenable to large-scale studies such as screenings for therapeutic compounds. In this study, a sensitive and quantitative enzyme-linked immunosorbant assay (ELISA) based on the detection of VP40 in lysed EBOV and VLPs is described. The assay is effective at detecting purified EBOV, EBOV from non-human primate serum and liver homogenate, and Ebola VLPs. Using this assay, the relative contributory effects of various EBOV proteins in VLP formation were also determined.

2. Materials and methods

2.1. cDNA constructs and antibodies

pWRG7077 plasmids containing cDNA for EBOV GP and VP40 were described previously (Bavari et al., 2002). cDNA for EBOV Zaire NP, VP30, VP35, and VP24 was cloned similarly into pWRG7077 vector. Monoclonal antibody AE11 was a gift of Dr. Kevin Anderson (United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, MD) and rabbit polyclonal antibodies against Ebola VP40 was described previously (Panchal et al., 2003). Polyclonal anti-VP40 antibody was generated by immunizing rabbits with a peptide corresponding to the N-terminal 15 amino acids of VP40 protein. Sera from convalescent EBOV-infected guinea pigs were used as the polyclonal anti-EBOV antibody.

2.2. Bacterial expression and purification of EBOV VP40

The VP40 gene was cloned in pET16b vector that introduced a His6-fusion at the N-terminus. The cell lysate from one liter of *Eschericia coli* expressing His-VP40 was resuspended in two volumes of extraction buffer (20 mM sodium phosphate buffer, pH 7.5, 100 mM NaCl, 5% glycerol, and 20 mM imidazole) and supplemented with complete protease inhibitor and EDTA, according to the manufacturer's instructions (Roche, Indianapolis, IN) per gram wet weight of cells. The cell lysate was digested with lysozyme (1 mg/ml) for 30 min on ice, adjusted to 5 mM $MgCl_2$, and treated with 10 U of benzonase/ml for an additional 20 min. The sample was adjusted to 500 mM NaCl, sonicated to lyse cells (verified by microscopic examination), centrifuged at 27,000 × *g* for 30 min, filtered (0.8 mm), and applied at 0.5 ml/min to a 5 ml chelating sepharose column (Amersham, Piscataway, NJ) charged with nickel and equilibrated with extraction buffer in 500 mM NaCl (buffer A). The column was washed with 10 column volumes of buffer A and protein eluted over a 100 ml gradient from 20 to 400 mM imidazole in buffer A. Fractions were analyzed by SDS-PAGE and appropriate fractions were pooled. The samples were then dialyzed against a buffer containing 20 mM sodium phosphate, 1 mM β-mercaptoethanol, 200 mM NaCl, and 10% glycerol.

2.3. Enzyme-linked immunosorbant assay

Immulon HB 96-well plates (Thermo Electron, Milford, MA) were coated with AE11 monoclonal mouse anti-VP40 antibodies using 1 μg per well diluted in 100 μl of phosphate-buffered saline (PBS) for 12 h. The plates were washed three times with PBS plus 0.02% Tween (PBST). PBST containing 5% milk was added to each well for 2 h to block non-specific binding sites. After binding, plates were washed three times for 5 min using PBST. Cellular extracts were prepared by disrupting cells on ice for 20 min in buffer containing:

50 mM Tris pH 7.5, 150 Mm NaCl, 1.5% Triton, and protease inhibitor cocktail (Pierce, Rockford, IL). Cell-culture supernatants or purified viruses were treated with 1.5% Triton X100 before being loaded into wells. Cellular extracts or culture supernatants were loaded using indicated amounts of sample per well in 1% milk PBST and rocked for 3 h. The samples were removed and the wells washed three times for 5 min. Rabbit polyclonal anti-VP40 antibodies (USAMRIID) were added at a 1:200 dilution in PBST/5% milk and incubated for 1 h. The plate was washed again and anti-rabbit HRP-conjugated antibodies were added at a 1:500 dilution in PBST for 30 min. The plate was washed three times for 5 min with PBST and once for 5 min with PBS. Horseradish peroxidase (HRP) substrate was added to each well and absorbance at 650 nm was read at 5 min intervals until the signal was well within the linear range of the assay.

2.4. Transfections and cell-culture

293T cells were maintained and grown in Dulbecco's Modified Eagle Medium (DMEM-GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). Cells were split before transfection into six-well plates at 500,000 cells per well in DMEM without antibiotics, and grown overnight. Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and varying amounts of purified plasmid DNA according to the manufacturer's instructions. Transfection mixtures were layered onto cells and incubated for 48 h before harvesting cell-culture supernatants and cells. Culture supernatants were clarified by centrifugation at 3000 rpm for 5 min in a micro-centrifuge. Clarified supernatants were either used directly or frozen at −80 °C until needed.

2.5. EBOV infection

Vero-E6 cells were infected at a multiplicity of infection (MOI) of 0.5 with EBOV-Zaire for 50 min at 37 °C, 5% CO_2 in BSL4 containment. Non-adsorbed virus was removed from cells by washing monolayers twice with PBS followed by adding fresh complete medium for the length of the incubation period. Supernatants were harvested and treated with Triton X100 at a final concentration of 1.5%. Cell lysates were also prepared as described above. All samples were inactivated by irradiation (10^7 R, ^{60}Co source) before being removed from the BSL4 laboratory.

2.6. Electron microscopy

VLPs were applied to 300-mesh, nickel electron microscopy grids pre-coated with formvar and carbon, treated with 1% glutaraldehyde in PBS for 10 min, rinsed in distilled water, and negatively stained with 1% uranyl acetate. Stained grids were examined with a JEOL 1200 EX transmission electron microscope at 80 kV.

2.7. Western blots

Protein experiments and Western blotting was performed by running samples on 4–12% polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferring the proteins to nitrocellulose (Bio-Rad, Hercules, CA) using a semi-dry transfer apparatus at 20 V for 20 min. Blots were probed for proteins with the antibodies indicated in the figures and developed by using HRP-conjugated secondary antibodies (MP Biomedicals, Irvine, CA) and chemiluminescent substrate (Pierce, Rockford, IL) on X-ray film (Kodak, Rochester, NY).

2.8. Immunostaining and confocal microscopy

Rabbit polyclonal anti-VP40 and mouse monoclonal anti-NP antibodies were used to visualize VP40 and NP in transfected 293T cells. Cells were grown on eight-well chamber slides, transfected, and after 24–48 h fixed in 4% formaldehyde, permeabilized in 0.1% Triton X100, and stained for examination by confocal microscopy. Alexa 488-conjugated, goat anti-mouse secondary antibody and Alexa 568-conjugated goat anti-rabbit antibody (1:500; Molecular Probes, Eugene, OR) were used to visualize the primary antibodies. Cell nuclei were labeled with Hoechst stain (Molecular Probes, Eugene, OR). Images were obtained with a Bio-Rad (Hercules, CA) 2000MP confocal/multiphoton system attached to a Nikon TE300 inverted microscope.

3. Results

3.1. Quantification of inactivated Ebola virus and Ebola virus-like particles using an ELISA

Experimentation with EBOV and its surrogate VLP model is critically dependent on a reliable quantitative assay for measuring particle release. Measuring EBOV is based currently on a functional plaque assay, which is highly sensitive but is very laborious and requires over a week to obtain results. In many molecular biology studies, a surrogate VLP release assay is used that relies on assaying VP40 or GP in culture supernatant by Western blot and enhanced chemiluminescence (ECL) analysis followed by densitometry of radiograms. However, the narrow window of linearity of ECL severely limits the quantitative utility of this approach. In order to circumvent these problems, we sought to develop a sensitive sandwich ELISA that could detect the EBOV matrix protein VP40 in culture supernatants. For this purpose, His-tagged EBOV Zaire VP40 was produced in *E. coli* and purified on a Ni^{2+} column. As shown in Fig. 1A, Coomassie blue stains of the purified protein indicated high degree of purity. Immunostaining with anti-VP40 antibody (AE11) verified the identity of this band as VP40 (Fig. 1A). This preparation contained a small amount of oligomeric VP40, as evident in unheated sample blotted with AE11 antibody (Fig. 1A). It must be noted that AE11 is very effective in

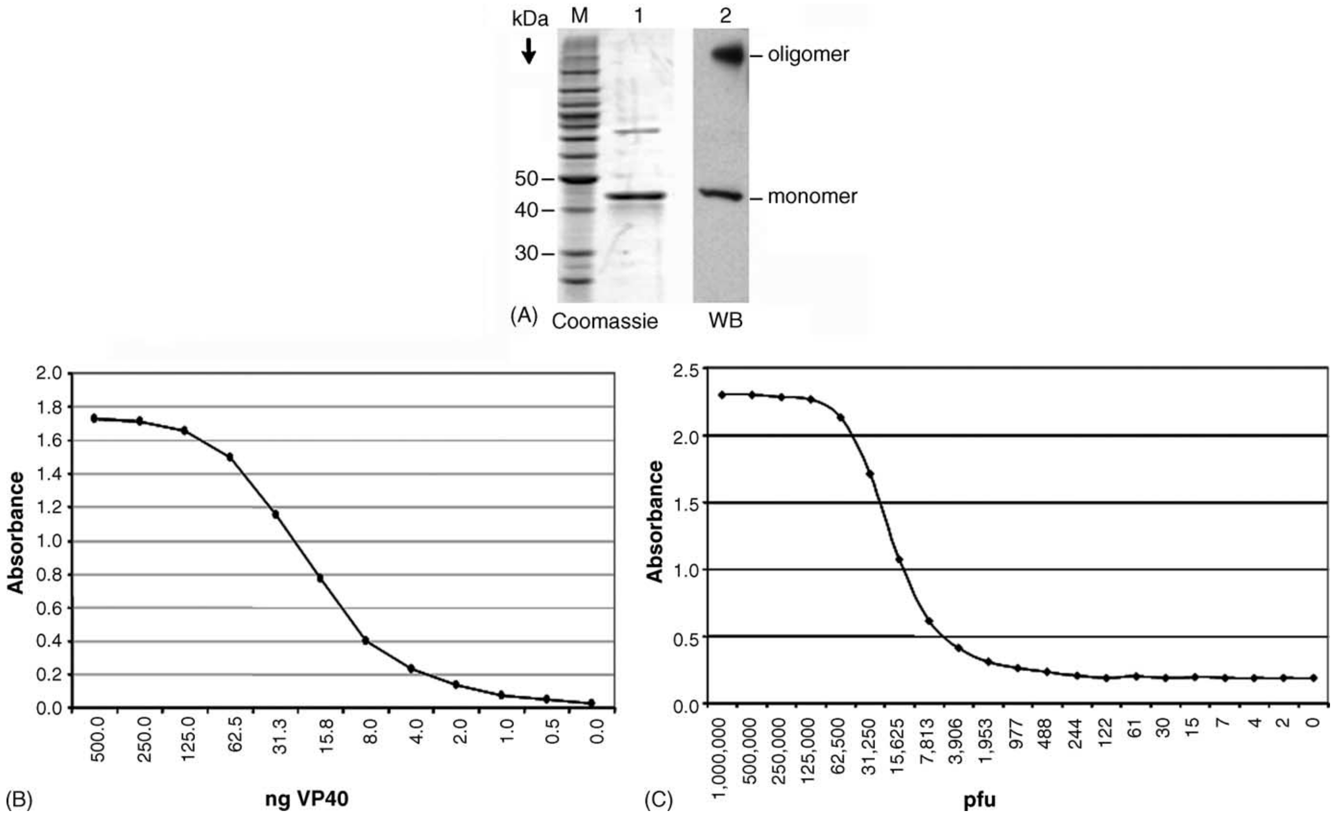


Fig. 1. Establishing an EBOV VP40 ELISA. (A) Coomassie staining of purified VP40 protein (lane 1). Immunoblotting of VP40 protein gel using anti-VP40 antibodies showing the monomeric and oligomeric forms of VP40 (lane 2). (B) Standard curve for VP40 ELISA generated using purified VP40 protein. (C) Standard curve for VP40 ELISA using inactivated EBOV lysed in 1.5% Triton X100. Capture antibodies were monoclonal mouse anti-VP40 and detection antibodies rabbit anti-VP40 polyclonal antisera.

detecting the oligomeric VP40 (our unpublished observation), therefore, the intensity of the oligomeric band in Fig. 1A, probably over-represents the oligomeric fraction of the preparation.

To establish an ELISA, mouse anti-VP40 monoclonal antibodies (AE11) were used to bind the plate and a rabbit polyclonal antibody elicited against the C-terminal 15 amino acids of VP40 (Panchal et al., 2003) for detection. Details of the assay are described in Section 2. A standard curve was generated with purified full-length EBOV VP40. As shown in Fig. 1B, purified VP40 was detectable down to about 2 ng, equivalent to 0.05 pmole. The assay displayed a linear range of about 10–100 ng, and the curve plateaued at about 250 ng (Fig. 1B). Next, the ability of this assay to detect authentic EBOV was tested. Irradiated viral particles of known quantity were lysed in 1.5% Triton X100 to expose VP40. Using this ELISA, it was possible to detect the virus with a sensitivity of about 500–1000 plaque-forming units (PFU), and a linear range between 5×10^3 and 1.0×10^5 PFU (Fig. 1C).

The ability of this assay to detect VLPs released from 293 T-cells transfected with EBOV VP40 and GP was next evaluated. As shown in Fig. 2A (top panel), VLPs could be detected in a volume of >2–3 μl of cell-culture supernatant out of a total of 1000 μl of cell-culture medium in a six-well culture dish. Cellular lysates were also tested and VP40 was detectable in less than 1 μl of lysate, corresponding to fewer than 5000 cells (Fig. 2B, top panel). The linear range for detecting VLPs in cell-culture medium was between 6 and 100 μl (0.6–10%) of supernatant, and for cellular lysates, the linear range was between 3 and 50 μl, corresponding to 15000–150,000 cells (Fig. 2). Concurrent Western blot analysis from the same samples demonstrated that the ELISA was much more sensitive, more quantitative, and had a broader linear range than immunoblotting (Fig. 2, lower panels).

3.2. Detection of EBOV in cell-culture supernatants of EBOV-infected cells

The assay was also tested for detecting EBOV in culture supernatants. Vero-E6 cells were infected with EBOV Zaire (MOI: 0.5) and viral replication was monitored concurrently by ELISA and plaque assay at several time points. As shown in Fig. 3A, ELISA of lysed virus in 50 μl of the supernatant displayed a linear range during the first 72 h of infection. In contrast, the cell-associated VP40 (tested in 10 μl of lysate) reached a plateau after 48 h. Fig. 3B shows the replication curve of EBOV in the same experiment performed with the

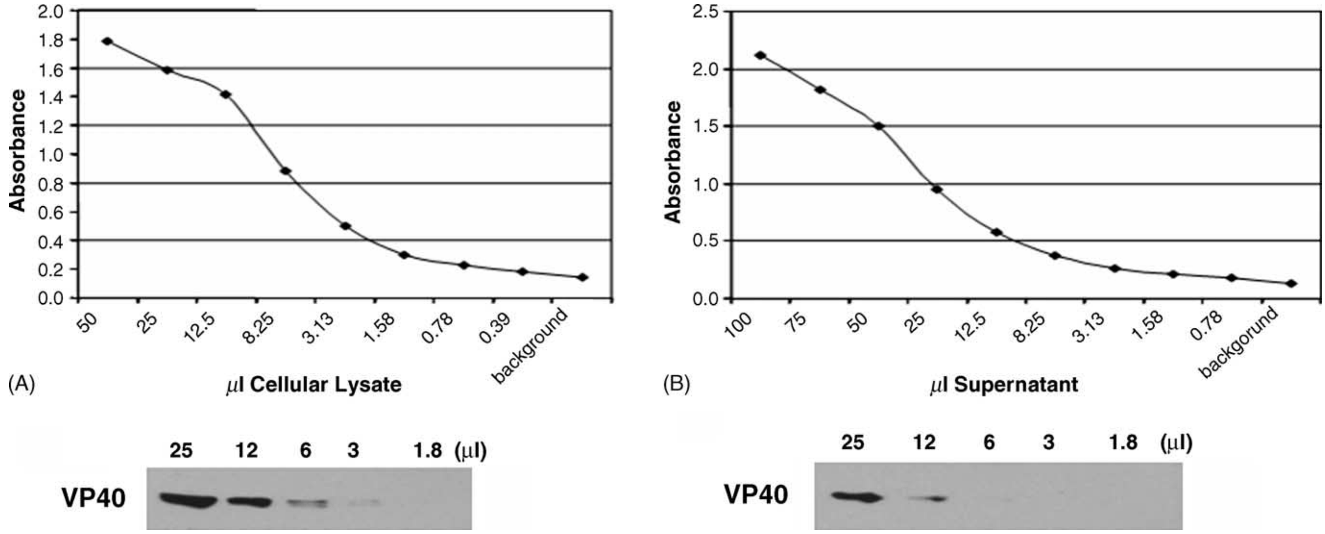
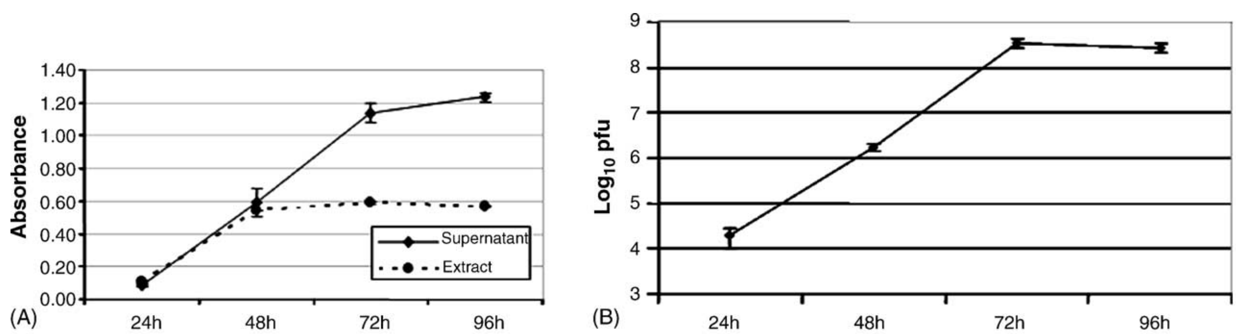


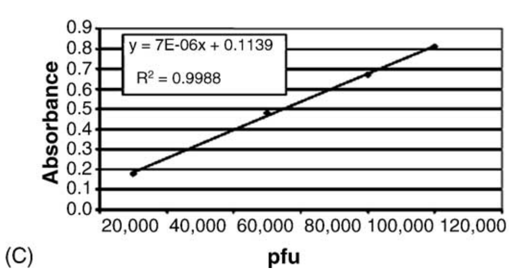
Fig. 2. Detection of VLPs in cell-culture supernatants and cellular lysates. (A) Different amounts of cellular lysates from cell-cultures transfected with plasmids encoding GP and VP40 were assayed to determine the linear range of the assay and limit of detection. (B) Clarified cell-culture supernatants were lysed in 1.5% Triton X100 and ELISA was performed as in (A). Western blot analyses of similar volumes of cell-culture supernatant and extract are shown in lower panels to compare the sensitivity of the two techniques.

conventional plaque assay. Using purified inactivated virus, an internal standard curve was generated to correlate the PFU of the tested samples from the ELISA data (Fig. 3C). As shown in Fig. 3D, the PFU s determined from ELISA data correlated well with the plaque assay. These results indicate that this ELISA can be used reliably as a rapid assay to quantitate EBOV in in vitro studies. Given that the ELISA can be carried out within a few hours in BSL2, this assay is an attractive alternative to plaque enumeration for rapid and large-scale evaluations in in vitro studies on Ebola virus release and testing of potential inhibitors.

3.3. Detection of EBOV in serum and tissues of EBOV-infected animals

In order to evaluate the potential of this assay as a backup diagnostic test, liver homogenate, and plasma samples from non-human primates that had been infected with EBOV were





Titer

	24h	48h	72h	96h
Plaque Assay	1.8×10^4 pfu/ml	1.0×10^6 pfu/ml	3.4×10^8 pfu/ml	2.7×10^8 pfu/ml
ELISA	3.5×10^4 pfu/ml	1.4×10^6 pfu/ml	5.5×10^7 pfu/ml	1.4×10^8 pfu/ml

(D)

Fig. 3. Measurement of EBOV in culture supernatants. (A) 293T cells were infected with EBOV and cell-culture supernatants and cell pellets were harvested at times indicated. Supernatants were treated with 1.5% Triton X100 and along with cellular lysates assayed to determine the viral VP40 content. (B) Plaque assays were performed on cell-culture supernatants from the experiment in (A). (C) An internal standard curve was generated using purified inactivated EBOV to correlate ELISA reading with PFUs. (D) Comparison of viral titers determined either directly (plaque assay) or interpreted from the ELISA standard curve.

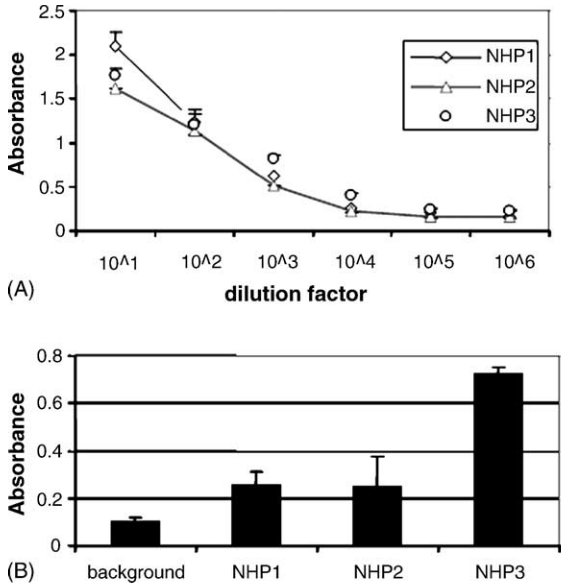


Fig. 4. Detection of EBOV in non-human primate samples. (A) Ebola-infected non-human primate liver homogenate (A) or plasma samples (B) were analyzed using VP40 ELISA.

analyzed. The assay readily detected EBOV in dilutions of liver homogenate (Fig. 4A). The assay also detected VP40-containing EBOV from infected non-human primate plasma samples; although, the assay was not as sensitive in plasma as in liver homogenate (Fig. 4B). It was concluded there was likely a component of the plasma that partially inhibited virus detection. Nonetheless, the ability to detect VP40 from both liver homogenate and plasma shows that, while this assay was insufficiently sensitive to diagnose early stage disease, it could be used to rapidly detect viremia in the course of animal studies.

3.4. *Application of VP40 ELISA to study the contribution of EBOV proteins to VLP release*

It has been shown that the presence of GP increases the efficiency of VP40 vesicular release (Bavari et al., 2002; Noda et al., 2002). During the preparation of this manuscript, Licata et al. (2004) also reported that coexpression of NP increases VLP production and release in VP40 expressing cells. However, the relative effect of each protein in stimulating VLP production has not been accurately determined in a quantitative manner. Immunofluorescence studies indicated that, when expressed alone, NP was associated with globular structures in the cytoplasm (Fig. 5A). However, in cells coexpressing EBOV VP40, a fraction of NP was redirected to the plasma membrane in close association with VP40 (Fig. 5A), suggesting that NP may be released with VP40 vesicles. Immunoblotting demonstrated that NP could be detected in the supernatant of cells expressing GP, VP40, and NP (Fig. 5B). VLPs produced by these cells were purified by sucrose gradient centrifugation and analyzed by immunoblotting. This experiment demonstrated that NP co-migrated with VP40 on the sucrose gradient, further supporting its association with VLPs (Fig. 5C). These data indicated the ability of NP to bud from the cells in the presence of VP40.

To evaluate the contribution of NP and other viral proteins to VLP release in a quantitative manner, optimal conditions for VLP formation were determined. Titrating different amounts of GP and VP40 plasmids in the transfection mix resulted in a bell-shaped curve, with 0.2 μg of each plasmid (for 1.75 cm plate) being optimal for VLP release (data not shown). Using these conditions as baseline, the effect of coexpression of NP on the efficiency of VLP release was studied. 293T cells were transfected with various combinations of GP, VP40, and NP, and cells and supernatants were harvested 48 h after transfection. ELISA was then carried out using cellular lysates and cell-culture supernatants. As shown in Fig. 5D, GP and NP, when individually transfected into cells with VP40, increased VLP production to about three-fold and co-transfection of all three plasmids further augmented the VLP release by up to five- to six-fold. Electron microscopy of the supernatants of cells transfected with the three plasmids revealed a large number of filamentous structures (Fig. 5E).

The nucleocapsid of EBOV consists of a complex of NP, L, VP35, and VP30 that encompass the RNA genome (Feldmann and Kiley, 1999). It has been reported that VP35 and NP, when expressed in presence of VP24, are sufficient for the formation of filamentous particles (Huang et al., 2002). Therefore, it was possible that coexpression of nucleocapsid components may improve the VLP release. The effects of VP35, VP30, and VP24 on VP40 VLP release were examined and it was found that none of these proteins had a significant effect on VLP production when transfected with VP40 alone (data not shown). However, when these plasmids were co-transfected with GP, VP40, and NP, there was a significant increase in VLP production (Fig. 6). While VP24 alone had only a minor effect on VLP release, VP30 and VP35 increased VLP production by about 50 and 130–150%, respectively. Combining VP30, VP24 or both with VP35 did not significantly change the efficiency of VLP release (Fig. 6). Because the presence of nucleocapsid components clearly enhanced the VLP release, we also asked if the presence of negative-strand RNA with EBOV flanking sequences would further increase VLP release. For this purpose, we used a recently reported RNA polymerase I (Pol-I)-based minigenome plasmid (Groseth et al., 2005). Expression of this plasmid produces a Pol-I transcript with EBOV leader and trailer sequences in viral RNA orientation that can be packaged into viral particles. However, repeated experiments did not demonstrate any significant change in the level of VLP release upon expression of the minigenome (data not shown), suggesting that the nucleocapsid structures that contribute to VLP release are stable in the absence of packageable RNA, which is consistent with a previous report (Huang et al., 2002). Taken together, these findings indicate that the nucleocapsid proteins NP, VP30, and VP35 can enhance significantly the release of EBOV VLPs.

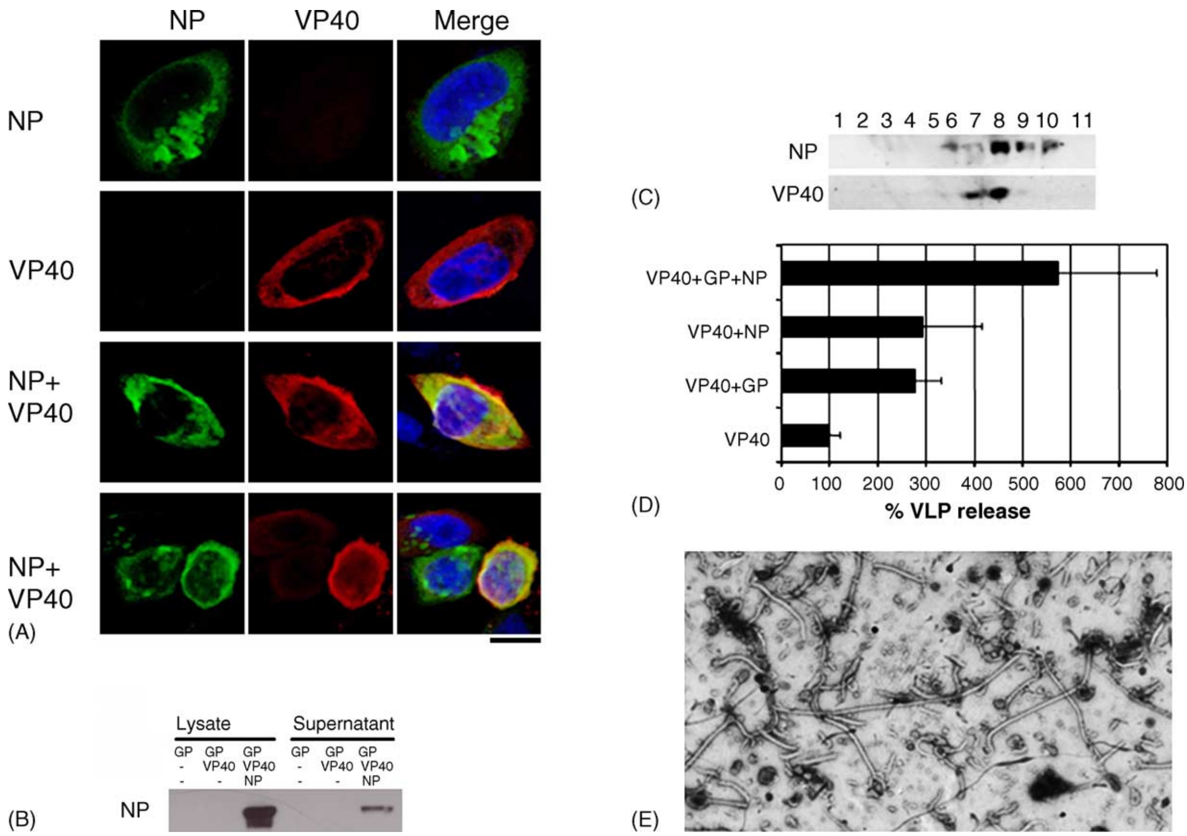


Fig. 5. NP is re-directed to the cell membrane by VP40 and is incorporated into VLPs. (A) 293T HEK cells were transfected with NP, VP40 or NP + VP40. Cells were fixed and immunostained for NP (green) and/or VP40 (red). Nuclei were stained blue (Hoechst dye). Yellow indicates regions of colocalization. (B) Immunoblot analysis of cellular lysates and cell-culture supernatants showing that NP is released into the supernatant in cells expressing NP, VP40, and GP. (C) VLPs in cell-culture supernatants were pelleted through a sucrose cushion and fractionated over a 10–60% sucrose gradient. Fractions were collected and immunoblotted for NP and VP40. (D) VLP release was analyzed by determining the ratio of VP40 in the culture supernatant to VP40 content of cellular lysates when transfected with VP40, VP40 + GP, VP40 + NP or VP40 + GP + NP. VP40 VLP release was set at 100%. (E) Representative electron micrograph of VLPs generated by cells transfected with VP40 + GP + NP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

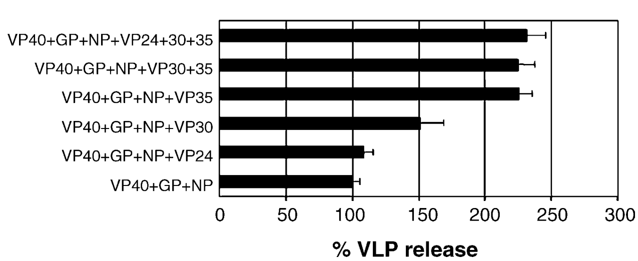


Fig. 6. Effects of additional Ebola proteins on VLP release. 293T HEK cells were transfected with the indicated plasmids and cellular lysates and cell-culture supernatants were analyzed using ELISA to determine the efficiency of VLP release by determining the ratio of VP40 in the culture supernatant to VP40 content of cellular lysates. Values from VP40 + GP + NP transfections were set at 100%.

4. Discussion

Although filoviruses have been studied for the past three decades, knowledge about the viral replication cycle and how viral proteins interact with cellular factors to coordinate viral assembly and release is very limited. Research on the molecular mechanisms of filovirus life cycle has been hampered by the restrictions of biocontainment laboratories as well as cumbersome virus detection assays. Described here is a quantitative ELISA that can be used to study molecular mechanisms of viral assembly and budding as well as virus–host cell interactions. Using this assay, the effect of therapeutic and experimental manipulations on EBOV replication can be rapidly measured, thus significantly enhancing research in the field. While this assay is not sufficiently sensitive to be used as a diagnostic tool, it can be very useful to rapidly detect viremia in the course of animal studies, as viremic animals normally have high titers well above the detection limit of this assay.

This assay was used successfully to determine the relative contribution of different EBOV proteins to VLP release, demonstrating its applicability in Ebola research. Although VP40 alone can drive the formation of filamentous VLPs that are released from cells (Timmins et al., 2001), our data clearly demonstrate that optimal VLP formation and release requires additional viral proteins, which is consistent with a recent report (Licata et al., 2004). These findings indicate that VP40 may be recruiting NP to the site of budding. While NP was associated entirely with inclusion-like bodies in the cytoplasm when expressed alone, coexpression of VP40 redirected a fraction of NP to the plasma membrane (Fig. 5). Consistent with this observation, NP was released to the supernatant of VP40/NP-expressing cells and co-migrated with VP40 on a sucrose gradient (Fig. 5). ELISA analysis showed that the expression of NP increased significantly the release of VP40 and VP40/GP VLPs. Colocalization of VP40 and NP in intact cells (Fig. 5), and a previous report showing a physical association between the two proteins (Licata et al., 2004) and the consequent release of NP-containing VLPs suggest that this may be the key interaction linking the nucleocapsid and envelope assembly processes. It is also interesting that a smaller fraction of VP40 showed some degree of colocalization with cytoplasmic NP (Fig. 5). This property of Ebola VP40 appears to be a common feature of negative-strand RNA viruses and may be critical for viral release (Aman et al., 2003; Garoff et al., 1998). NP alone does not appear to form filamentous structures. Therefore, the enhancement of VLP release cannot be explained by an instructive effect of NP toward generation of a filamentous structure. Consistent with this no nucleocapsid-like core structures in NP-containing VLPs were observed (Fig. 5E). It is possible that the physical association between VP40 and NP increases the stability of the resulting VLPs. However, more studies are needed to delineate the mechanism of NP-mediated enhancement of VLP release.

Huang et al. (2002) showed that formation of nucleocapsid requires NP, VP35, and VP24, with VP24 playing a less well defined, presumably catalytic role in the process. Cells expressing these three proteins form large number of filamentous structures that fail to bud from the cells (Huang et al., 2002). VP30 has also been shown to colocalize with NP inclusion bodies in the absence of other viral proteins. We, therefore, studied the effect of each of the nucleocapsid proteins on VLP release. Neither VP30, VP35, nor VP24 alone had an effect on VLP release when individually coexpressed with VP40. In contrast, VP30 and VP35 individually and in combination increased substantially VLP release from cells expressing GP, VP40, and NP. These data clearly demonstrate that the efficiency of VLP release can be significantly increased by adding nucleocapsid proteins, possibly due to an instructive effect of the filamentous nucleocapsid on particle formation.

It was demonstrated recently that VLPs containing GP and VP40 alone convey protective immunity to EBOV in rodent models (Warfield et al., 2003). It is possible that incorporating the additional Ebola proteins into VLPs would further improve the immunogenicity of the particles. DNA vaccines expressing NP have shown protective immunity in rodents (Vanderzanden et al., 1998; Wilson et al., 2001; Wilson and Hart, 2001). Furthermore, vaccinations with combination of filovirus GP and NP protected non-human primates against EBOV and MARV infections (Hevey et al., 1997, 1998; Sullivan et al., 2000, 2003). A protective immune response against filoviruses requires robust cellular and humoral immune responses (Feldmann et al., 2003). NP has been shown to induce a strong cytotoxic T-cell response (Wilson and Hart, 2001). Therefore, adding NP to a VLP-based vaccine may significantly improve vaccine efficacy. While other viral proteins such as VP40, VP24, VP30, and VP35 expressed by a Venezuelan encephalitis virus (VEE)-based replicon are immunogenic when administered to mice, their protective effects are less robust and seem to depend on the strain of mice used (Wilson et al., 2001). Nevertheless, adding nucleocapsid proteins, as shown here, can improve the VLP yield and may also enhance the stability of the structures. Therefore, it is reasonable to hypothesize that VLPs made of GP, VP40, and components of nucleocapsid may prove to be a more versatile and efficacious vaccine against EBOV infections in NHPs and humans. While our data indicate that VP35 can contribute to an increase yield of VLP, a lesser augmentation of VLP release was also achieved by VP30 (Fig. 6). While mouse studies have shown some protective effect by Ebola VP35 expressed in replicons, VP35 is suggested to be an interferon antagonist presumably assisting the virus to escape the innate immune response (Basler et al., 2000) and its presence in a vaccine preparation may be of concern. More studies are needed to evaluate if the presence of VP35 in a VLP-based vaccine would improve or reduce its efficacy. Furthermore, the absence of the RNA polymerase L in these VLPs rules out the safety concern about the possibility of a catastrophic recombination event. We were also able to generate VLPs using VP30 and VP35 from the Reston strain of EBOV combined with other proteins from Zaire strain (data not shown). This indicates the possibility of generating a vaccine that may convey protective immunity against multiple viral strains. Studies are currently underway in our laboratory to evaluate the immunogenicity and vaccine efficacy of VLPs containing GP, VP40, and the nucleocapsid proteins.

Development of a rapid Ebola VLP/virus release assay that can be carried out in BSL2 laboratories has also important implications for developing therapeutics. It has been demonstrated that this assay can be adapted and utilized in a high throughput format (data not shown). A high throughput EBOV release assay can be used to screen chemical libraries to identify lead antiviral compounds. It is becoming increasingly clear that cellular factors play a critical role in filovirus assembly and budding suggesting that such factors may be valuable therapeutic targets (Aman et al., 2003). The cell-based nature of this assay allows us to identify compounds targeting viral and/or cellular proteins involved in viral replication.

Taken together, the results described above have important implications both for vaccine and therapeutics development against Ebola virus hemorrhagic fever. In addition, this rapid ELISA was shown to be a powerful tool for basic research aimed at understanding the molecular mechanisms of EBOV assembly and budding.

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References

Aman, M.J., Bosio, C.M., Panchal, R.G., Burnett, J.C., Schmaljohn, A., Bavari, S., 2003. Molecular mechanisms of filovirus cellular trafficking. Microbes Infect. 5 (7), 639–649.

Basler, C.F., Wang, X., Muhlberger, E., Volchkov, V., Paragas, J., Klenk, H.D., Garcia-Sastre, A., Palese, P., 2000. The Ebola virus VP35 protein functions as a type I IFN antagonist. Proc. Natl. Acad. Sci. U.S.A. 97 (22), 12289–12294.

Bavari, S., Bosio, C.M., Wiegand, E., Ruthel, G., Will, A.B., Geisbert, T.W., Hevey, M., Schmaljohn, C., Schmaljohn, A., Aman, M.J., 2002. Lipid raft microdomains: a gateway for compartmentalized trafficking of Ebola and Marburg viruses. J. Exp. Med. 195 (5), 593–602.

Dessen, A., Volchkov, V., Dolnik, O., Klenk, H.D., Weissenhorn, W., 2000. Crystal structure of the matrix protein VP40 from Ebola virus. Embo J. 19 (16), 4228–4236.

Feldmann, H., Jones, S., Klenk, H.D., Schnittler, H.J., 2003. Ebola virus: from discovery to vaccine. Nat. Rev. Immunol. 3 (8), 677–685.

Feldmann, H., Kiley, M.P., 1999. Classification, structure, and replication of filoviruses. Curr. Top. Microbiol. Immunol. 235, 1–21.

Feldmann, H., Klenk, H.D., 1996. Marburg and Ebola viruses. Adv. Virus Res. 47, 1–52.

Freed, E.O., 2002. Viral late domains. J. Virol. 76 (10), 4679–4687.

Garoff, H., Hewson, R., Opstelten, D.J., 1998. Virus maturation by budding. Microbiol. Mol. Biol. Rev. 62 (4), 1171–1190.

Groseth, A., Feldmann, H., Theriault, S., Mehmetoglu, G., Flick, R., 2005. RNA polymerase I-driven minigenome system for Ebola virus. J. Virol. 79, 4425–4433.

Hevey, M., Negley, D., Geisbert, J., Jahrling, P., Schmaljohn, A., 1997. Antigenicity and vaccine potential of Marburg virus glycoprotein expressed by baculovirus recombinants. Virology 239 (1), 206–216.

Hevey, M., Negley, D., Pushko, P., Smith, J., Schmaljohn, A., 1998. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. Virology 251 (1), 28–37.

Huang, Y., Xu, L., Sun, Y., Nabel, G.J., 2002. The assembly of Ebola virus nucleocapsid requires virion-associated proteins 35 and 24 and posttranslational modification of nucleoprotein. Mol. Cells 10 (2), 307–316.

Jasenosky, L.D., Neumann, G., Lukashevich, I., Kawaoka, Y., 2001. Ebola virus VP40-induced particle formation and association with the lipid bilayer. J. Virol. 75 (11), 5205–5214.

Licata, J.M., Johnson, R.F., Han, Z., Harty, R.N., 2004. Contribution of Ebola virus glycoprotein, nucleoprotein, and VP24 to budding of VP40 virus-like particles. J. Virol. 78 (14), 7344–7351.

Licata, J.M., Simpson-Holley, M., Wright, N.T., Han, Z., Paragas, J., Harty, R.N., 2003. Overlapping motifs (PTAP and PPEY) within the Ebola virus VP40 protein function independently as late budding domains: involvement of host proteins TSG101 and VPS-4. J. Virol. 77 (3), 1812–1819.

Noda, T., Sagara, H., Suzuki, E., Takada, A., Kida, H., Kawaoka, Y., 2002. Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. J. Virol. 76 (10), 4855–4865.

Panchal, R.G., Ruthel, G., Kenny, T.A., Kallstrom, G.H., Lane, D., Badie, S.S., Li, L., Bavari, S., Aman, M.J., 2003. In vivo oligomerization and raft localization of Ebola virus protein VP40 during vesicular budding. Proc. Natl. Acad. Sci. U.S.A. 100 (26), 15936–15941.

Scianimanico, S., Schoehn, G., Timmins, J., Ruigrok, R.H., Klenk, H.D., Weissenhorn, W., 2000. Membrane association induces a conformational change in the Ebola virus matrix protein. Embo J. 19 (24), 6732–6741.

Sullivan, N.J., Geisbert, T.W., Geisbert, J.B., Xu, L., Yang, Z.Y., Roederer, M., Koup, R.A., Jahrling, P.B., Nabel, G.J., 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature 424 (6949), 681–684.

Sullivan, N.J., Sanchez, A., Rollin, P.E., Yang, Z.Y., Nabel, G.J., 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature 408 (6812), 605–609.

Swenson, D.L., Warfield, K.L., Kuehl, K., Larsen, T., Hevey, M.C., Schmaljohn, A., Bavari, S., Aman, M.J., 2004. Generation of Marburg virus-like particles by co-expression of glycoprotein and matrix protein(1). FEMS Immunol. Med. Microbiol. 40 (1), 27–31.

Timmins, J., Scianimanico, S., Schoehn, G., Weissenhorn, W., 2001. Vesicular release of Ebola virus matrix protein VP40. Virology 283 (1), 1–6.

Vanderzanden, L., Bray, M., Fuller, D., Roberts, T., Custer, D., Spik, K., Jahrling, P., Huggins, J., Schmaljohn, A., Schmaljohn, C., 1998. DNA vaccines expressing either the GP or NP genes of Ebola virus protect mice from lethal challenge. Virology 246 (1), 134–144.

Warfield, K.L., Bosio, C.M., Welcher, B.C., Deal, E.M., Mohamadzadeh, M., Schmaljohn, A., Aman, M.J., Bavari, S., 2003. Ebola virus-like particles protect from lethal Ebola virus infection. Proc. Natl. Acad. Sci. U.S.A. 100 (26), 15889–15894.

Warfield, K.L., Perkins, J.G., Swenson, D.L., Deal, E.M., Bosio, C.M., Aman, M.J., Yokoyama, W.M., Young, H.A., Bavari, S., 2004a. Role of natural killer cells in innate protection against lethal Ebola virus infection. J. Exp. Med. 200 (2), 169–179.

Warfield, K.L., Swenson, D.L., Negely, D.L., Schmaljohn, A., Aman, M.J., Bavari, S., 2004b. Marburg virus-like particles protect guinea pigs from lethal Marburg virus infection. Vaccine 22 (25–26), 2495–2502.

Watanabe, S., Watanabe, T., Noda, T., Takada, A., Feldmann, H., Jasenosky, L.D., Kawaoka, Y., 2004. Production of novel Ebola virus-like particles from cDNAs: an alternative to Ebola virus generation by reverse genetics. J. Virol. 78 (2), 999–1005.

Wilson, J.A., Bray, M., Bakken, R., Hart, M.K., 2001. Vaccine potential of Ebola virus VP24, VP30, VP35, and VP40 proteins. Virology 286 (2), 384–390.

Wilson, J.A., Hart, M.K., 2001. Protection from Ebola virus mediated by cytotoxic T lymphocytes specific for the viral nucleoprotein. J. Virol. 75 (6), 2660–2664.